AD_____

GRANT NUMBER DAMD17-96-1-6180

TITLE: Peroxisomal Oxidation in Normal and Tumoral Human Breast

PRINCIPAL INVESTIGATOR: Dr. Gillian Small

CONTRACTING ORGANIZATION: Mount Sinai School of Medicine
New York, New York 10029

REPORT DATE: October 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1998	Final (13 Sep 96 - 13 Sep 98)

4. TITLE AND SUBTITLE Peroxisomal Oxidation in Normal and Tumoral Human Breast

5. FUNDING NUMBERS
DAMD17-96-1-6180

6. AUTHOR(S)
Dr. Gillian Small

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Mount Sinai School of Medicine
New York, NY 10029

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The peroxisomal enzyme catalase protects aerobic organisms from free radical damage by converting hydrogen peroxide (H_2O_2) to molecular oxygen and water before it can decompose to form the highly reactive hydroxyl radical. In this manner catalase plays a central role in protecting against cellular oxidative damage. In humans, changes in catalase activity have been implicated in aging and in a number of disease states including cancer. We hypothesized that reduced catalase could potentially lead to an excess of H_2O_2 produced by peroxisomal oxidative reactions, which may then leak into the cell and cause DNA damage. To test this hypothesis we have examined the mRNA levels of catalase and peroxisomal fatty acyl-CoA oxidase in a variety of human breast samples and in breast cancer cell lines and in normal tissue and non-immortalized cells in culture. In addition, we previously found that the human **P**eroxisome **P**roliferator-**A**ctivated **R**eceptor (hPPAR), a transcription factor involved in the regulation of peroxisomal ß-oxidation enzymes, is highly expressed in human mammary tissue. Therefore we also compared the levels of hPPAR with those of catalase and acyl-CoA oxidase. Using the polymerase chain reaction to quantitate the expression of the genes encoding each of these proteins, we found that the levels were similar in the human breast tumor samples and in normal controls, with the exception that we were unable to detect hPPAR in an estrogen receptor positive carcinoma cell line.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	15
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

✓ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

S M. S 10/15/98

PI - Signature Date

Table of Contents

Introduction

Peroxisomes are organelles that play an essential role in cell respiration and lipid metabolism. They contain several hydrogen peroxide-producing oxidases, and catalase which decomposes the hydrogen peroxide to water and molecular oxygen. The presence of peroxisomes has been confirmed in all human tissues looked at using cytochemical and/or biochemical techniques. There is very little information on peroxisomes and their functionality in human tumorous tissues, however reduced levels of catalase activity have been reported in colon carcinomas (4) human liver tumors (8) and in human epitheliomas (7). Concerning human breast tissue, a study was performed in 1992 demonstrating that the specific activities of some peroxisomal enzymes were diminished in tumoral breast epithelial cells when compared to controls (1). These investigators demonstrated that catalase activity in breast neoplastic tissues belonging to grade III (most fully progressed) were significantly lower than in adjacent normal tissues, while no differences were found in the number of peroxisomes. In this study the activities of some peroxisomal oxidases (including fatty acyl-CoA oxidase) were reduced, while others were unchanged. A simultaneous deficiency in peroxisomal ß-oxidation and of catalase activity could lead to the over-production of lipid peroxides, which are also implicated in carcinogenesis (2,10).

At the onset of our study the information available regarding the fluctuation in peroxisomal enzyme activities in humans raised the possibility that low catalase levels could be a factor in DNA damage and thus, in tumor formation or progression. There was no information regarding the relationship of the three proteins we proposed to study (catalase, acyl-CoA oxidase and the peroxisome proliferator-activated receptor (PPAR)) in human tumors. Breast tumors are an ideal model for us to test the hypothesis that variations in the ratios of these proteins may be involved in the tumorigenic process.

Our goal is to understand the role of peroxisomal metabolism in human mammary tissue. Initially we decided to concentrate on measuring the activities of catalase and acyl-CoA oxidase in human normal and tumorigenic breast tissue in order to understand whether there is any systematic correlation between the activities of these enzymes and the degree of tumorigenicity. We intend to also measure the expression levels of hPPAR in these tissues in order to determine whether hPPAR levels correlate with acyl-CoA oxidase activity. We would expect this to be the case if this receptor is regulating acyl-CoA oxidase in human mammary tissue. We will further determine whether there is any correlation between hPPAR expression and tumor formation. By investigating these parameters we intend to establish their possible involvement in breast tumor initiation/progression.

Experiments and Results

1) To determine levels of catalase in human breast tissue and human breast tumor samples.

To test the hypothesis that low catalase levels in combination with normal or increased levels of peroxisomal oxidative enzymes may lead, or contribute, to DNA damage in breast tumors our initial approach was to measure catalase activity in normal breast epithelial tissue and in tumorigenic tissue samples. However, we found that the catalase activity in human breast tumor samples was extremely low, and at the limits of detection by our assay. Details of these results were given in the annual report (1996-97).

Due to the limits of measuring both catalase and acyl-CoA oxidase by standard assay procedures, we turned to a more sensitive approach using the polymerase chain reaction.

Conclusions

Our studies for objective one demonstrated that the levels of peroxisomal catalase and acyl-CoA oxidase in human breast tumor samples are very low, and outside the limit of quantitative analysis by standard assays. Therefore we adopted an alternative approach and established conditions for accurate quantitation of gene expression using the polymerase chain reaction.

2) Quantitation of human peroxisomal enzyme gene expression using the polymerase chain reaction.

A polymerase chain reaction (PCR)-based method has previously been used to quantitate the expression levels of low abundance genes (3,5,6). In this method RNA is first isolated from human samples or cultured cells and is subsequently converted to cDNA by reverse transcription using random hexamers. This DNA can then be amplified by PCR and the products generated can be quantitated by various means. While this method is extremely sensitive, one must be aware of the exponential nature of the polymerase chain reaction and the potential problems of using this method for quantitative purposes. Therefore we adopted the method of Horikoshi et al. in which the relative, rather than the absolute, levels of gene expression are determined by comparing the ratio of PCR product generated by amplification of the target DNA and an endogenous internal standard gene in separate reactions (3). Samples of 1 μg of RNA from control cells were subjected to reverse transcription and the cDNA was then used in a polymerase chain reaction using primers designed to amplify regions of human ß-actin, catalase or acyl-CoA oxidase. The primers were end-labeled with

γ ^{32}P-ATP using T4 kinase. Samples of the reaction mixtures were then electrophoresed through a 10% acrylamide gel. The gel was dried, exposed to a phosphorImager screen and analyzed using Imagquant software. Levels of catalase and acyl-CoA oxidase were normalized to levels of ß-actin for each sample.

Establishing linearity of the reaction

The increase in the amount of PCR product as a function of the starting amount of DNA is described by the equation:

$$N = N_0 (1+eff)^n$$

where N_0 is the number of copies of the starting DNA

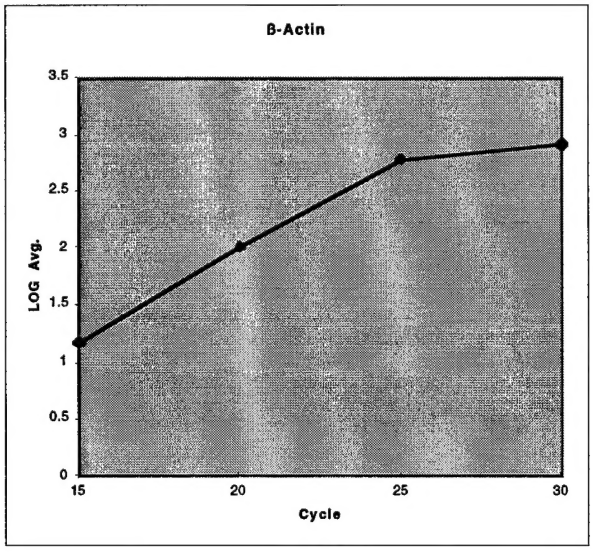
N is the amount of DNA after *n* cycles of amplification

eff is the efficiency of the amplification

(9).

According to this equation, even though the PCR reaction products increase exponentially, the amplified DNA at any value of *n* is linearly proportional to the amount of starting DNA. However, the efficiency of the PCR becomes lower with increasing extent of reaction due to consumption of primers and build-up of inhibitory phosphate levels. Therefore the linearity of the PCR product curves depend on the cycle number. In order to obtain an indication of the range of linear amplification for each gene of interest as a function of cycle number we performed a series of time course experiments. The resultant curves were linear in the middle region between 20 and 25 cycles in each case (Figure 1). All subsequent experiments were carried out using 25 cycles.

(a)



(b) (c)

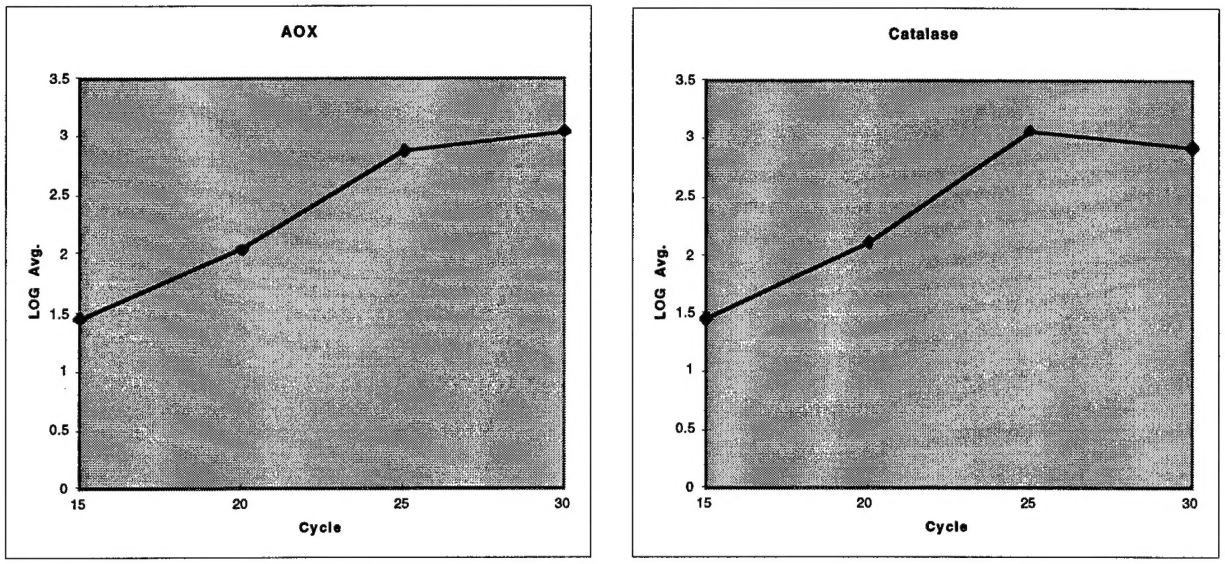


Figure 1. Optimization of the linearity curve for PCR of (a) actin, (b) acyl-CoA oxidase and (c) catalase cDNA. PCR products formed are plotted as a function of cycle number.

Use of reverse transcription PCR to measure the expression of genes encoding peroxisomal proteins in human breast tumor cell lines

The cell types shown in Table 2 were grown and harvested by Dr. Mira-y-Lopez who is collaborating with us on this project.

Table 2. Normal and carcinoma human breast cell lines used in this study.

Cells	Source
1) RM 12.5.94p2	Normal human breast cells derived from reduction mamoplasty
2) MT3V1-7	SV40 Immortalized normal luminal human breast epithelial cells
3) 184B5	Benzypyrene immortalized normal human breast epithelial cells
4) ZR75-1	Carcinoma cell line, estrogen receptor positive (ER+)
5) MDA-MB-468	Carcinoma cell line ER-
6) T47D	Carcinoma cell line ER+
7) BT20	Carcinoma cell line ER-
8) HS578T	Carcinoma cell line ER-
9) MDA-MB-231	Carcinoma cell line ER-

Approximately 5 X 10^6 cells were harvested by scraping and pelleting and were then snap-frozen at -80°C. Prior to use the cells were thawed and resuspended in 350 µl of lysis buffer. RNA was then extracted using a Qiagen RNeasy total RNA kit according to the protocol of the manufacturer. Approximately equal amounts of RNA were derived from each cell type (Figure 2).

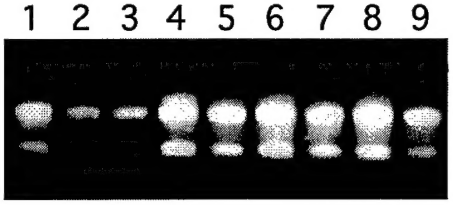


Figure 2. Ethidium bromide-stained gel of RNA isolated from cell samples 1 to 9 as shown in Table 2.

Samples of 1 µg of RNA were subjected to reverse transcription using the Gibco Superscript preamplification system. 10% of the reverse transcribed mixture was used as a template for each subsequent PCR reaction. The DNA primers for each reaction were designed to amplify fragments of ß-actin, hPPAR, catalase and acyl-CoA oxidase respectively and were labeled as described above. Initially we tested one control and one tumor cell line (numbers 1 and 7 from Table 2). PCR products of the expected size were visible on an ethidium bromide-stained gel for all fragments except hPPAR (Figure 3).

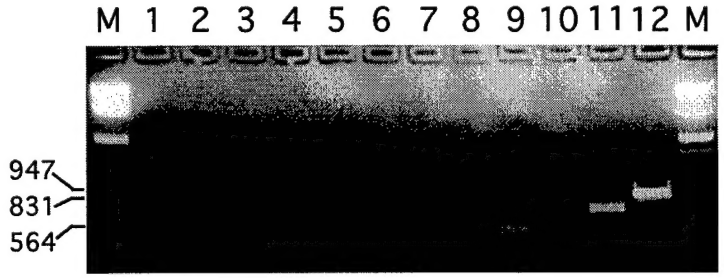


Figure 3. PCR products generated in the presence (lanes 1-4 and 9 to 12) and absence (lanes 5 to 8) of reverse transcriptase. PCR products were generated with primers to ß-actin (lanes 1, 5 and 9), hPPAR (lanes 2, 6 and 10), acyl-CoA oxidase (lanes 3, 7 and 11) and catalase (lanes 4, 8 and 12). The products were resolved in a 1% agarose gel and stained with ethidium bromide. M = molecular weight standards.

Comparison of acyl-CoA oxidase and catalase levels in human breast cell lines

The relative expression of acyl-CoA oxidase and catalase with respect to ß-actin was determined in three control samples (1 to 3 in Table 2) and two tumor cell lines (3 to 5 in Table 2) (Figure 4). The relative expression of acyl-CoA oxidase in control cells was fairly constant while the expression of catalase showed a three-fold variability (Table 3). The relative expression of both genes was higher in the tumor cell lines tested.

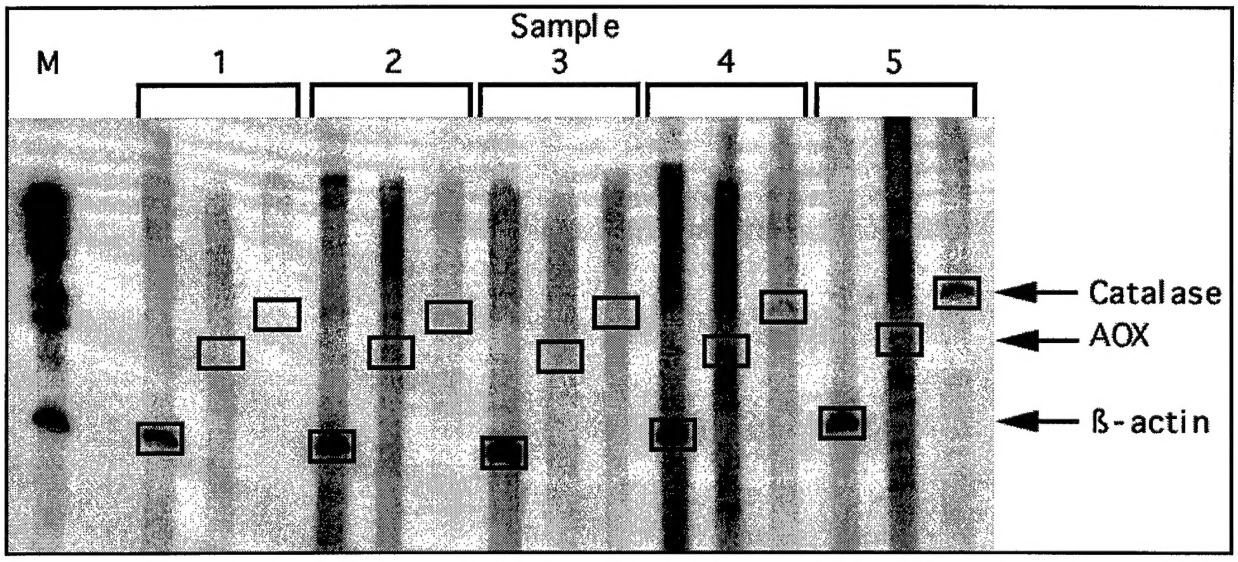


Figure 4. PCR products from amplification of ß-actin, acyl-CoA oxidase and catalase cDNA from samples 1 to 5 of Table 2. The PCR products were resolved in 10% polyacrylamide and the dried gel was exposed to a phosphorImager screen. M = radiolabeled DNA standards.

Table 3. Quantitation of PCR products shown in Figure 4.

Sample	Relative Aox expression	Relative catalase expression	Ratio Aox : catalase
1	17.4	5.8	3:1
2	23.6	8.46	3:1
3	16.25	18.05	1:1
4	45.2	21.15	2:1
5	45.3	52.7	1:1

In our mid-project summary we reported that we were unable to detect hPPAR in the human breast cells lines using the standard PCR protocol. For this reason we adopted a more sensitive approach, in which radiolabeled nucleotides were included in the PCR reaction mixture so that the radioactive

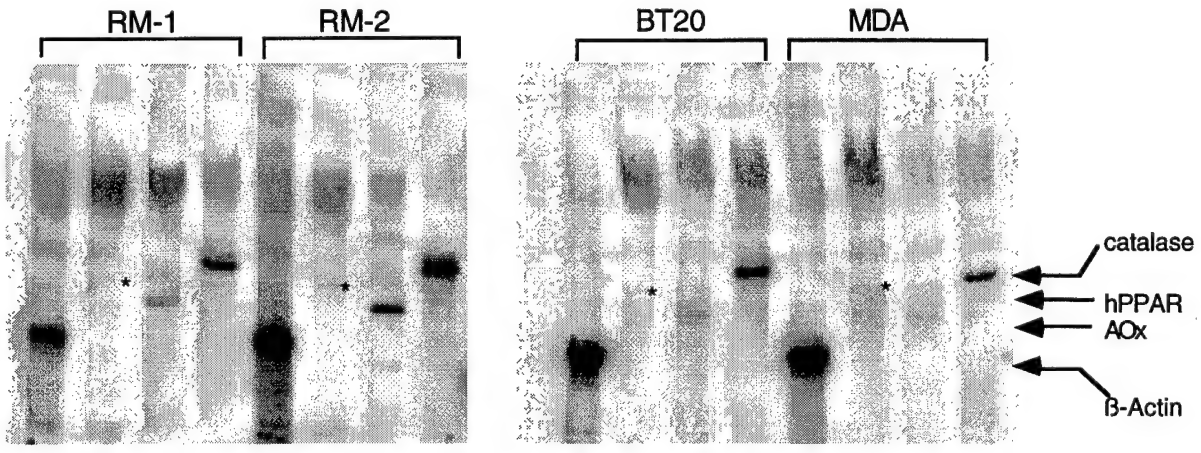
signal is amplified. This protocol is described in the next section (objective 3), and additional results comparing the levels of acyl-CoA oxidase and catalase expression are included in this section.

3). Comparison of the expression levels of hPPAR and peroxisomal acyl-CoA oxidase and catalase in breast cancer cell lines and in non-immortalized cells.

In order to detect hPPAR mRNA levels we adopted the RT-PCR approach of Murphy et al. (6), in which [^{32}P]dCTP is included in the PCR reaction. These assays were performed with the following cell lines (supplied by Dr. Mira-y-Lopez):

Cells	Source
RM 11.3.97	Normal human breast cells derived from reduction mamoplasty
RM 12.15.95	Normal human breast cells derived from reduction mamoplasty
BT20	Carcinoma cell line - estrogen receptor negative
MDA-MB-468	Carcinoma cell line - estrogen receptor negative
ZR75	Carcinoma cell line - estrogen receptor positive
MT3V1-7	SV40 immortalized normal luminal human breast epithelial cells

Essentially, 1 µg of RNA were subjected to reverse transcription using the Gibco Superscript preamplification system (GibcoBRL). 10% of the reverse transcribed mixture was used as a template for each subsequent PCR reaction. The DNA primers for each reaction were designed to amplify fragments of ß-actin, hPPAR, catalase and acyl-CoA oxidase respectively. The PCR was performed in 100 µl reaction mixtures which contained 10 µCi of [^{32}P]dCTP as described (6). Linearity of the procedure was established as described previously (see results section for objective 2). Using this more sensitive approach we were able to detect hPPAR in all of the samples except for the ZR75 cells (hPPAR is indicated by asterisks in Figure 5).



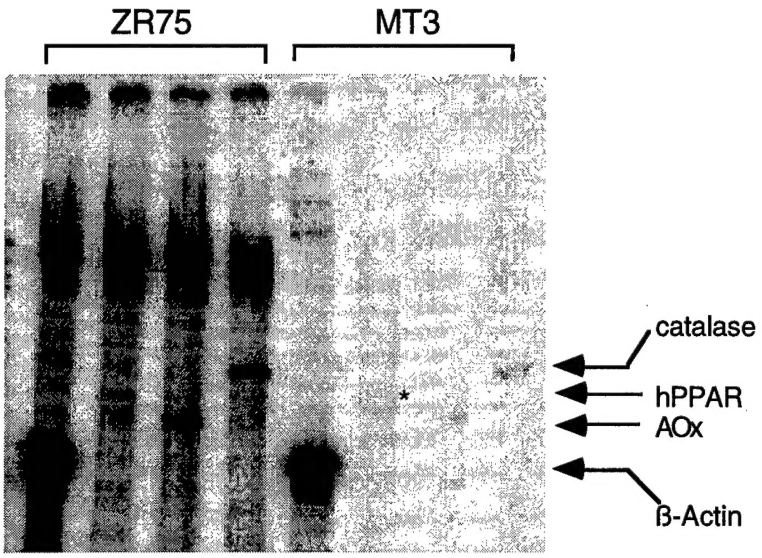


Figure 5. PCR products from amplification of β-actin, acyl-CoA oxidase (Aox), hPPAR and catalase cDNA from the human cell lines indicated. The PCR products were resolved in 10% polyacrylamide, and the dried gel was exposed to a phosphorImager screen. Intensity of the bands was quantitated using Imagequant software.

The RT-PCR reaction was performed at least three times with each cell line, and the results compared. hPPAR was not detected in ZR75 cells, even when the gels were overexposed (as shown in Figure 6).

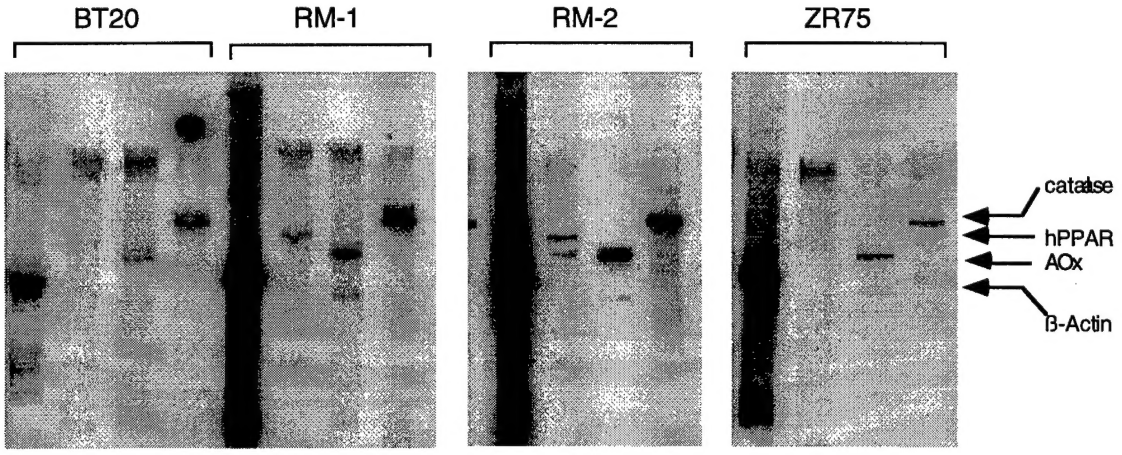


Figure 6. PCR products from amplification of β-actin, acyl-CoA oxidase (Aox), hPPAR and catalase cDNA from the human cell lines indicated. The products were analyzed as described for Figure 5.

The relative expression of acyl-CoA oxidase (AOx), catalase and hPPAR was determined using ß-actin as a standard, as described previously. These numbers were then used to compare the ratios of AOx : hPPAR and catalase : hPPAR respectively. Since these are relative numbers, the data from each experiment could not be compared, however the respective ratios were compared and found to be reproducible. We also compared the AOx : catalase ratio for each cell line. A table showing numbers from two separate experiments is shown below.

Cell Line	AOx : hPPAR	Catalase : hPPAR	AOx : catalase
RM -1 exp. 1	2.65 : 1	8.27 : 1	0.32 : 1
exp. 2	2.39 : 1	9.00 : 1	0.26 : 1
RM-2 exp. 1	5.44 : 1	10.20 : 1	0.53 : 1
exp. 2	4.32 : 1	11.16: 1	0.38 : 1
MT3 exp. 1	2.48 : 1	2.14 : 1	1.16 : 1
exp. 2	1.35 : 1	2.64 : 1	0.51 : 1
BT20 exp. 1	1.98 : 1	9.70 : 1	0.20 : 1
exp. 2	1.78 : 1	9.76 : 1	0.18 : 1
MDA exp. 1	0.77 : 1	8.48 : 1	0.09 : 1
exp. 2	0.56 : 1	9.76 : 1	0.18 : 1
ZR75 exp. 1	-	-	0.87 : 1
exp. 2	-	-	1.12 : 1

Conclusions

We have established conditions for accurate quantitation of gene expression using the polymerase chain reaction, and have analyzed the ratio of expression of acyl-CoA oxidase to catalase, and acyl-CoA oxidase to hPPAR in cells from reduction mamoplasty as well as from an immortalized cell line and various tumor cell lines. In these experiments we did not find any significant differences between normal and carcinoma cell lines, with the exception that hPPAR was **not** detectable in ZR75 cells (an estrogen receptor positive cell line), but **is** detectable in the two estrogen receptor negative carcinoma cell lines that we studied. At this stage we do not understand the relevance of this finding, and clearly need to study more cell lines (both ER positive and ER negative) .

References

1. **Bouhtoury, F. E., J-M. Keller, S. Colin, R. Michel, R. Parache, and M. Dauca.** 1992. Peroxisomal enzymes in normal and tumoral human breast. J. Pathol. **166**:27-35.

2. **Cerutti, P. A.** 1985. Prooxidant states in tumor promotion. Science **227**:375-381.

3. **Horikoshi, T., K. D. Danenberg, T. H. W. Stadlbauer, M. Volkenandt, L. C. C. Shea, K. Aigner, B. Gustavsson, L. Leichman, R. Frosing, M. Ray, N. W. Gibson, C. P. Spears, and P. V. Danenberg.** 1992. Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in human tumors using the plymerase chain reaction. Cancer Res. **52**:108-116.

4. **Keller, J-M., S. Cable, F. E. Bouhtoury, S. Heusser, C. Scotto, L. Armbruster, E. Ciolek, S. Colin, J. Schilt, and M. Dauca.** 1993. Peroxisome through cell differentiation and neoplasia. Biol. Cell **77**:77-88.

5. **Kinoshita, T., J. Imamura, H. Nagai, and K. Shimotohno.** 1992. Quantification of gene expression over a wide range by the polymerase chain reaction. Anal. Biochem. **206**:231-235.

6. **Murphy, L. D., C. E. Herzog, J. B. Rudick, A. T. Fojo, and S. E. Bates.** 1990. Use of the polymerase chain reaction in the quantitation of *mdr-1* gene expression. Biochemistry **29**:10351-10356.

7. **Rabilloud, T., D. Asselineau, C. Miquel, R. Calvayrac, M. Darmon, and M. Vuillaume.** 1990. Deficiency in catalase activity correlates with the appearance of tumor phenotype in human keratinocytes. Int. J. Cancer **45**:952-956.

8. **Roels, F. and S. Goldfischer.** 1979. Cytochemistry of human catalase. J. Histochem. Cytochem. **27**:1471-1477.

9. **Wang, A. M., M. V. Doyle, and D. F. Mark.** 1989. Quantitation of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA **86**:9717-9721.

10. **Wang, M-Y. and J. G. Liehr.** 1995. Lipid hydroperoxide-induced endogenous DNA adducts in hamsters: Possible mechanism of lipid hydroperoxide-mediated carcinogenesis. Arch. Biochem. Biophys. **316**:38-46.

Personnel paid by grant:

Dr. Igor Karpichev - Postdoctoral fellow.